Variation in the Photochemical Reactivity of Thymine in the DNA of B. subtilis Spores, Vegetative Cells and Spores Germinated in Chloramphenicol.*+

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Running Head: Photochemistry of Spore DNA

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Abstract.

The chief photoproduct of thymine produced in U.V. irradiated vegetative cells is the cyclobutane-type dimer while in spores very little of this dimer is produced (maximum yield 2.6% of thymine) but a new photoproduct is produced in high yield (maximum yield of 28.4% of thymine). This difference in photochemical response appears to be due, at least in part, to a difference in hydration of the DNA. The photochemistry of isolated DNA irradiated in solution is similar to that of DNA in irradiated vegetative cells, but differs markedly from that of isolated DNA irradiated dry. The yield of cyclobutane-type dimer is much reduced in isolated DNA irradiated dry but a new photoproduct is produced which is chromatographically similar to the spore photoproduct. The yield of this photoproduct, however, is never as great as that obtained in irradiated spores.

Since the sum of the maximum yield of cyclobutane-type thymine dimer (2.6%) and of the new photoproduct (28.4%) is very close to the maximum value expected for thymine dimerization in B. subtilis (33.9% of the thymine residues are adjacent), it is therefore suggested that the new spore photoproduct is a dimer of thymine with a new and unique structure. On a column of molecular sieve material (Sephadex-G10), the spore photoproduct elutes in a region intermediate between the cyclobutane-type dimer and monomeric thymine.

Although the photochemistry of thymine in the DNA of spores differs markedly from that for vegetative cells, several lines of evidence make it seem doubtful that the enhanced resistance of spores to U.V. relative to that of vegetative cells can be explained solely on the basis of this difference in the photochemistry of DNA thymine.

The photochemistry of the DNA thymine of spores germinated in the presence of chloramphenicol is very similar to that of normal vegetative cells. Except for hydration, the physical state of the DNA is probably not otherwise altered by germination in the presence of chloramphenicol since DNA replication is prevented by the presence of chloramphenicol. These results argue further that the unique photochemistry of spores is probably due at least in part to the hydration state of the DNA and suggest that the DNA of spores does not exist in a "super-coiled" state as it does in polyoma virus.

Introduction.

The photochemical sensitivity of DNA is, in part, a function of its physical state. The rate of formation of thymine dimers in heat-denatured DNA is about twice that for native DNA (15,22). The yield of thymine dimers is greatly depressed if DNA is irradiated dry (11; and present report). Spores are less sensitive to U.V. irradiation than are vegetative cells (10). The DNA of spores is thought to be in a more arid state than in vegetative cells (8). A recent report has also indicated that the photochemistry of DNA thymine is markedly different in spores and in vegetative cells of B. megaterium (6).

The DNA of spores of Bacillus subtilis that have been germinated in the presence of chloramphenicol (and therefore the initiation of DNA synthesis has been prevented) does not function as a primer for DNA polymerase action until treated with deoxyribonuclease for a short time (25). The DNA of polyoma virus is present in a "super-coiled" state which does not assume the double stranded circular shape until one single strand break has been introduced into the molecule by pancreatic deoxyribonuclease or chemical reducing agents (21). If the DNA of spores were in a "super-coiled" state, this might explain why it does not function properly as a primer for DNA polymerase activity until a change in its physical state is brought about by preliminary treatment with deoxyribonuclease.

This hypothesized change in the physical state of the spore DNA brought about by treatment with deoxyribonuclease might be followed as a change in the photochemical reactivity of the DNA and might be a more sensitive criterion than physical measurements upon isolated DNA since the DNA need not be isolated prior to the exposure to U.V. We therefore compared the photochemical response of the DNA thymine of B. subtilis vegetative cells, spores, and spores germinated in the presence of chloramphenicol with and without subsequent treatment with

deoxyribonuclease in an effort to determine at what stage in the germination cycle the physical state of the DNA spores is altered in the hope that it may help to explain (1) why the DNA of spores germinated in the presence of chloramphenicol does not function as a primer for DNA polymerase action until treated with deoxyribonuclease for a short time, and (2) determine if there is a photochemical explanation for the enhanced U.V. resistance of spores.

Methods.

A mutant of B. subtilis 168 that requires thymine, indole and leucine (MY2Y1U2) was used. For the preparation of thymine-C-14 labeled spores, cells were grown in synthetic medium (5) supplemented with thymine-2-C-14 (25.2 mc/mM, Calbiochem) carrier-free at 0.4 μc/ml, and L-leucine and L-tryptophan at 50 μgm/ml. A 250 ml culture was shaken in a 2-liter flask at 37° for 4 days. Sporulation was followed by microscopic examination. The spores and residual cells were harvested by centrifugation. The cells were lysed with 1 mg/ml lysozyme for 60 min. at 37°C followed by treatment with 1% sodium lauryl sulfate at 37°C for 30 min. The spores were harvested and washed 5 times with distilled water. The specific activity of the spores was 18,300 cpm/unit of O.D. at 650 mμ.

The procedure for the germination of spores in the presence of chloramphenicol has been described (25). Under these conditions no incorporation of leucine-C-14 into protein could be detected and no replication of DNA took place.

The techniques used for the ultraviolet irradiation (2537A) of cells, their subsequent hydrolysis in trifluoroacetic acid and chromatographic analysis have been described (17).

Cells were grown in mineral medium (2) supplemented with 2 μgm/ml of thymine-2-C-14 (24.3 mc/mM; Calbiochem), 50 μgm/ml of L-leucine and L-tryptophan and glucose at 0.5%. Late log phase cells were irradiated in growth medium for the times indicated, made to 5% trichloroacetic acid (TCA) washed several times in cold 5% TCA, then several times with ethanol/ether (3/1) at room temperature and once at 60°C for 10 minutes, and then hydrolyzed.

Spores were irradiated in water and then processed as described for the cells.

To isolate DNA from vegetative cells, they were suspended in 3.5 ml of 0.05 M TRIS buffer at pH 8.1 and an equal volume of lysozyme-EDTA solution (1 ml of 2 mg/ml lysozyme, Worthington, plus 4 ml of 1 mg/ml EDTA) and incubated for 5 minutes at 37°; 7 ml of 4% sodium lauryl sulfate was then added, and the clear solution stirred for 60 minutes at room temperature. The procedure outlined by Smith (13) was followed subsequently. The isolated mixture of DNA and RNA was treated with ribonuclease (Worthington) at 0.36 mg/ml for 60 minutes at 37°, an equal volume of 4% sodium lauryl sulfate was added and the solution stirred at room temperature for 60 minutes. This was followed by the KCl treatment and alcohol precipitation steps in the DNA isolation procedure (13). The DNA was finally dissolved in water (4 $O.D._{260}$ units/ml) and had 1.14×10^5 cpm/$O.D._{260}$ unit. This solution was diluted approximately 1:3 for irradiation in solution. After irradiation, the DNA was precipitated with alcohol and acid hydrolyzed. DNA samples (0.25 ml of stock solution) were also evaporated to dryness in a vacuum desiccator and irradiated dry. These samples were taken up directly in trifluoroacetic acid for hydrolysis. A second preparation of DNA (Sample #2 in Table 3) was used at a concentration of 0.8 $O.D._{260}$ units/ml and had 1.83×10^5 cpm/$O.D._{260}$ unit (0.5 ml aliquots were dried for irradiation).

Experimental Results.

1. Photochemistry of Thymine-C-14 Labeled Spores and Vegetative Cells. About 45 ml of late log phase cells were irradiated with U.V. (2537A°) in growth medium for 90 minutes (4.7×10^4 ergs/mm^2) and 10 ml of spores (3×10^7/ml) were irradiated in water for 60 minutes (3.1×10^4 ergs/mm^2) and processed as described under methods.

The photochemistry of thymine in the DNA of spores is markedly different from that for vegetative cells of Bacillus subtilis. Almost the only photoproduct of thymine that was produced in the cells was the thymine dimer (Table 1). Very little thymine dimer was formed in the irradiated spores, however, another photoproduct of thymine was formed in large amount. This material had an R_f of 0.35 in the butanol/acetic acid/water (80/12/30) solvent [14] compared to the dimer at 0.28 and thymine at 0.60. Similar results have been observed for Bacillus megaterium [6].

2. Photochemistry of Thymine-C-14 Labeled Spores and Spores Germinated in Chloramphenicol. Normal spores and spores germinated in the presence of 100 μgm/ml chloramphenicol were irradiated for 90 minutes (4.7×10^4 ergs/mm^2), hydrolyzed and chromatographed.

The results for the normal spores (Table 2) are essentially the same as found in the previous experiment (Table 1). Very little thymine dimer was produced but a lot of the photoproduct at R_f 0.35 was produced. The results for the spores germinated in the presence of chloramphenicol, however, were very similar to those for vegetative cells (Table 1), that is, mostly thymine dimer was formed (Table 2).

Our expectation had been that the photochemistry of spores germinated in chloramphenicol would be identical to that for normal spores and would only change after the subsequent treatment with deoxyribonuclease. As shown above, the germination process itself was sufficient to alter the photochemistry. Treatment of the germinated spores with deoxyribonuclease prior to U.V. irradiation had no additional effect on the photochemistry of DNA.

3. Irradiation of DNA in vitro. The chief photoproduct of thymine that was produced when DNA was irradiated (4.7×10^4 ergs/mm^2) in solution was the thymine dimer (Table 3). When the DNA was irradiated while dry, the amount of thymine

dimer produced was greatly reduced (see also reference #11) and there was an increase in the amount of the 0.35 material produced. When DNA Sample #1 was irradiated dry, the major photoproducts chromatographed at or near the orgin. At the lowest dose of U.V. (2.3×10^4 ergs/mm^2) principally material at Rf 0.01 was formed but at the higher dose (4.7×10^4 ergs/mm^2) the amount of this material decreased and the material at Rf 0.04 showed a corresponding increase suggesting that the two materials are closely related. When DNA Sample #2 was irradiated dry almost no origin material was formed and the yield of dimer and Rf 0.35 material was increased. The DNA solution was somewhat more concentrated in Sample #1 (4 $O.D._{260}$ units/ml) as compared to Sample #2 (0.8 $O.D._{260}$ units/ml) and this may explain the difference in the amount of origin material formed. The two samples may also have been irradiated at a different humidity since dry Sample #1 was irradiated simultaneously with a sample in solution and dry Sample #2 was irradiated by itself. Drying DNA Sample #2 in 0.15 M NaCl (as compared to water used above) had only a slight effect upon its photochemistry (Table 3) (compare reference #6).

4. Attempts to Identify the Photoproduct of Thymine Produced in Spores.

A. Comparisons by Paper Chromatography.

The spore photoproduct (arising from thymine-2-C-14) was chromatographed in several solvents along with numerous marker materials in an attempt at identification. The results are in Table 5.

Chromatographically the spore photoproduct is rather similar to 5-hydroxyuracil (isobarbituric acid). It cannot be this compound since the photoproduct is formed with the same yield in irradiated spores labeled with thymine-methyl-C-14 or with thymine-2-C-14 (Table 4).

The spore photoproduct is also chromatographically rather similar to (but not identical with) 5-hydroxymethyluracil and also to the Type III isomer of the thymine dimer (Table 5). This isomer has the thymine rings in Cis position relative

to the cyclobutane ring but the methyl groups are Trans. The chief isomer of thymine produced in DNA (Type I) appears to be Cis in both considerations [23,24].

The evidence that the spore photoproduct cannot be the Type III cyclobutane-type thymine dimer is that the Type III dimer is converted back to thymine on reirradiation in solution whereas the spore photoproduct is not altered under these conditions (as evidenced by rechromatography after reirradiation).

Our chromatographic data allow us to conclude that the spore photoproduct is not identical with certain marker materials but does not allow us to establish the identity of the photoproduct.

B. What is the Maximum Yield of Photoproduct?

Since the spore photoproduct (R_f 0.35) shows no short wavelength reversal to thymine as compared to the cyclobutane-type thymine dimer (R_f 0.28), it is therefore possible that the spore product might not have a wavelength dependency for reaching a maximum yield of photoproduct as does the cyclobutane-type thymine dimer (for discussions of this phenomenon see references16,18). Early experiments (cited in Table 1) did in fact indicate that a much higher yield of thymine photoproduct was produced in spores (R_f 0.35 material) than in vegetative cells (thymine dimer, R_f 0.28) for a given dose of U.V.

For Bacillus subtilis 33.9% of the thymines are adjacent to each other [7]. If after very high doses of U.V. (2537A) the yield of the spore photoproduct (R_f 0.35) plateaued at this value it would be good indirect evidence that the spore photoproduct was a dimer of thymine. The results of experiments to measure the maximum percentage of the thymine converted to photoproducts versus dose of U.V. are given in Figure 1. The yield of spore photoproduct (R_f 0.35) plateaued at a value of 28.4%. The maximum yield of cyclobutane-type thymine dimer (Type I) was about 2.6%. This adds up to 31% which is very close to the theoretical limit (33.9%) for the dimerization of thymine in single strands of DNA in B. subtilis. We therefore offer the hypothesis that the spore photoproduct is a new and unique type of thymine dimer.

C. Determination of Molecular Size by Chromatography on Sephadex G-10.

We had observed that a distinction between thymine (molecular weight 126) and the cyclobutane-type thymine dimer (molecular weight 252) produced when thymine was irradiated in frozen solution could be made on the basis of their behavior on a short column of molecular sieve material Sephadex G-10 (Figure 2). The thymine dimer emerged first from the column with the peak centered at about tube #30 while the thymine was retarded by the column and emerged at tube #40. The Type III cyclobutane-type thymine dimer also emerges at tube #30 (19). When the spore photoproduct (R_f 0.35) was submitted to the column, it emerged at tube #36, which is about half way between the value for the monomer and the dimer. These results are therefore inconclusive in predicting the precise molecular weight of the spore photoproduct, although the results presented in Section 4B are suggestive that the product is a dimer of thymine.

Discussion.

The photochemistry of thymine in the DNA of bacterial spores is markedly different from that for vegetative cells. The chief photoproduct of thymine produced in vegetative cells is the cyclobutane-type thymine dimer (Type I). Very little of this type of thymine dimer is produced in irradiated spores but another photoproduct of thymine is produced in high yield. The maximum yield of this material in spores is about 28% and is very close to the value of 33.9% which is the percentage of thymines that are nearest neighbors (7) and therefore represents the maximum amount of thymine that could be dimerized within a single strand of DNA. This experiment suggests that the spore photoproduct is a dimer of thymine but since it shows no alteration after reirradiation in solution, it cannot be any of the five isomers of the cyclobutane-type thymine dimer that have been isolated (19,23). The experiment to prove the dimeric character of this new photoproduct by an evaluation of its physical size using a column of molecular sieve material, Sephadex G-10, was inconclusive except that

it showed that the spore photoproduct did not behave either as a cyclobutane-type dimer or as monomeric thymine but in fact showed intermediate properties. The spore photoproduct also chromatographs on paper in the butanol/acetic acid/water solvent to a position intermediate between the cyclobutane-type dimer (Type I) and monomeric thymine. The fact that the spore photoproduct is isolated after the spores have been hydrolyzed in trifluoroacetic acid at 155°C for 60 minutes speaks for its stability to acid. The alkaline stability of the photoproduct has not been investigated.

The cyclobutane-type dimer has been shown to be of biological importance in the U.V. inactivation of cells (for discussions of this see references 16, 18). It has been suggested that the greater resistance of spores to killing by U.V. as compared to vegetative cells may be due to the fact that little of this dimer is produced in irradiated spores (6). This explanation, however, has the disadvantage that it ignores the possible biological importance of the new photoproduct of thymine produced in high yield in irradiated spores. It has been reported that this photoproduct is lost from the spores during germination (6). If the spore photoproduct were repaired in situ and if this mechanism were more efficient than the "cut and patch" repair mechanism (4,9,12) shown to be operative on thymine dimers, this might explain the greater U.V. resistance of spores. However, if the new photoproduct is repaired by the same mechanism or with the same efficiency as the cyclobutane-type thymine dimer, then it is hard to see how the formation of this photoproduct in lieu of the cyclobutane-type thymine dimer could explain the U.V. resistance of spores.

E. coli cells are more sensitive to killing by U.V. when irradiated in frozen solution than they are when irradiated at room temperature (3,20). Although the total amount of thymine altered by a given dose of U.V. is not different whether the cells are irradiated at room temperature or at -79°C,

there is a decrease in the amount of cyclobutane-type thymine dimer formed with a concomitant appearance of a material that is refractory to reirradiation in solution and which behaves chromatographically similar to the spore photoproduct (20). If the spores are more resistant to killing by U.V. because of the formation of this photoproduct, then it is difficult to understand how E. coli cells can be more sensitive to killing by U.V. as a consequence of the formation of this compound. The increased sensitivity of frozen E. coli cells to killing by U.V. has been correlated with an increase in the amount of DNA crosslinked to protein (20).

The cell wall of a spore probably swells during germination and thus permits a better equilibration of the interior of the cell with exterior water. Except for hydration the physical state of spore DNA should not be altered by germination in the presence of chloramphenicol since this treatment prevents DNA replication (25). The photochemistry of the DNA thymine of spores germinated in the presence of chloramphenicol is very similar to that of normal vegetative cells, and it suggests that the unique photochemistry of spores may involve a difference in the hydration of DNA.

The photochemistry of isolated DNA irradiated in solution is very different from that of DNA irradiated dry but is similar to that of DNA in vegetative cells. This suggests that the DNA within vegetative cells is thoroughly hydrated. Since the photochemistry of the DNA of spores differs markedly from that of vegetative cells or DNA in solution but is more similar to that of isolated DNA irradiated as a dry film, it suggests that the DNA within a spore is in a dry, compressed state (see also reference #8). However, the yield of new photoproduct in dry DNA never reaches the yield that it does in spores, so dryness alone cannot explain the unique photochemistry of spores.

The failure of the DNA of spores that have been germinated in the presence of chloramphenicol to act as a primer for DNA polymerase until the spores have been treated for a short time with deoxyribonuclease is apparently not due to a "supercoiled" state of the DNA. The physical state of the DNA that regulates the formation of the cyclobutane-type thymine dimer between adjacent thymines in a single strand of DNA is not further altered after germination in chloramphenicol by a subsequent treatment with deoxyribonuclease. The failure of the DNA to act as a primer still remains to be explained and this problem is being further pursued.

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Table 1 - Per cent Production of Photoproducts of Thymine-2-C-14 in Cells and Spores of B. subtilis.

Photoproducts	Cells	Spores
Origin Area	0.1%	0.0%
Thymine Dimer (Rf 0.28) (Cyclobutane-type)	7.2%	2.7%
Rf 0.35	0.0%	11.4%

U.V. Dose: Spores, 3.1×10^4 ergs/mm^2; Cells, 4.7×10^4 ergs/mm^2

Chromatographic Solvent: n-Butanol/acetic acid/water (80/12/30) (Reference #14). See text for further details.

Table 2 - Per cent Production of Photoproducts of Thymine-2-C-14 in Spores and Spores Germinated in Chloramphenicol.

Photoproducts	Spores	Spores plus Chloramphenicol
Origin	0.5%	0.2%
Thymine Dimer (Rf 0.28) (Cyclobutane-type)	3.8%	6.2%
Rf 0.35	12.5%	1.2%

U.V. Dose: 4.7×10^4 ergs/mm^2 (2537A)

See text for further details.

Table 3 - The photochemistry of Thymine in DNA

Irradiated in Solution or Dry.

Photoproducts	Solution	Dry		Dry in 0.15 M NaCl
	(Sample #1)	(Sample #1)	(Sample #2)	(Sample #2)
Origin Area	0.4%	5.7%	0.3%	0.8%
Thymine Dimer (Rf 0.28) (Cyclobutane-type)	6.1%	1.4%	2.1%	3.8%
Rf 0.35	0.6%	1.4%	3.0%	2.9%

U.V. dose: 4.7×10^4 ergs/mm^2 (2537A^o)

Sample numbers refer to two different samples of B. subtilis DNA (See section on Methods).

Table 4 - Yield of Thymine Photoproducts in Spores

Labeled with Thymine-2-C-14 or Thymine-Methyl-C-14

Photoproducts	Thymine-2-C-14	Thymine-Methyl-C-14
Origin Area	0.4%	0.7%
Thymine Dimer (Rf 0.28) (Cyclobutane-type)	1.7%	2.4%
Rf 0.35	21.3%	21.0%

Dose of U.V.: 4.7×10^4 ergs/mm^2

Spores irradiated in water at about 10^7 spores/ml.

Note: These two samples of spores were from a different preparation than the one used in the experiments cited in Tables 1 and 2. Although these spores were prepared as described under Methods, their appearance was different from the first preparation in that they were much less pigmented (brown) than the first preparation. This difference in pigmentation may explain the greater yield of photoproduct per unit dose of U.V., but it is apparent the qualitative photochemistry is not significantly different from the first batch of spores (Tables 1 and 2).

Table 5. R_f Values

Compound	Chromatographic Solvent				
	A	B	C	D	E
Spore Photoproduct	.35	.21	.56	.49	.49
Uracil	.48	.34	--	.57[a]	--
Dihydrohydrouracil	.47	.28	--	--	--
Urea	.53	.28	--	--	--
5-Hydorxymethyluracil	.35	.27	.64	.48[a] .51	--
5-Formyluracil[c]	.44	.37	.59	.54[a] .56	--
5-Carboxyuracil	--	--	--	.08[a]	--
5-Hydroxyuracil	.36	.25	.56	.47	--
Thymine	.60	.55	.78	.68[a] .72	.59
Dihydrothymine	.61	.53	.79	.64	--
Uracil Dimer	.12	.03	--	--	--
Thymine-Uracil Dimer	.19	--	--	--	--
Thymine Dimer (Type I)[d]	.28	.12[b] .14	--	--	.49[b] .49
(Type III)[d]	.33[e]	.17[b][e]	.62[e]	.40[e]	.56[b][e]

Chromatographic Solvents:

A. n-Butanol/Acetic Acid/Water (80/12/30)

B. n-Butanol/Water (86/14)

C. Isopropanol/HCl/Water (68/15.5/16.5)

D. Sec. Butanol saturated with water

E. Isopropanol/NH_3/Water (7/1/2)

(a) Data of Alcantara and Wang [1]

(b) Data of Weinblum and Johns [23]

(c) Sample of 5-Formyluracil courtesy of S.Y. Wang.

(d) For a description of the isomeric forms of the thymine dimer, see reference #24.

(e) Data of Smith [19].

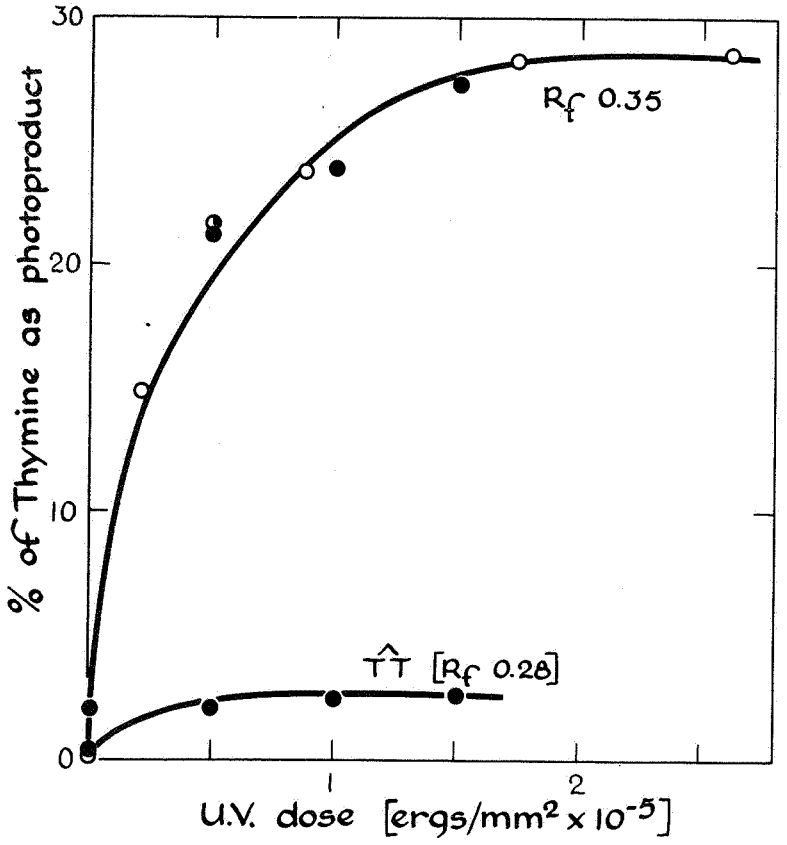
Legends to the Figures.

Figure 1. Dose Response Curves for the Production of Photoproducts of Thymine in DNA of U.V. Irradiated Spores of Bacillus subtilis.

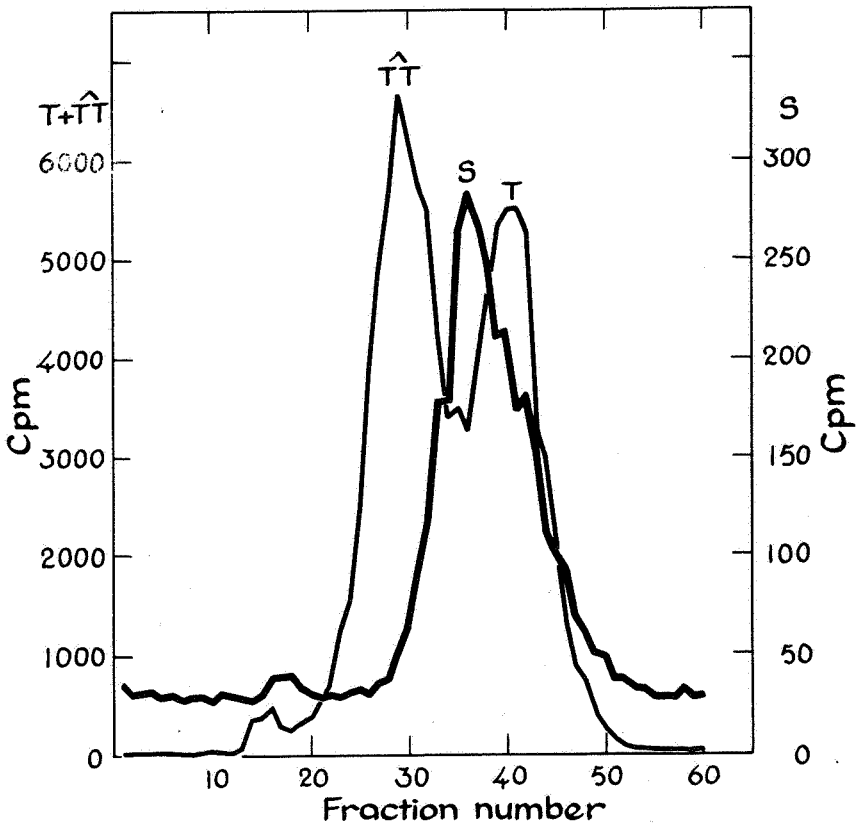
The thymine-2-C-14 labeled spores discussed in the footnote to Table 4 were used here. The different symbols indicate separate experiments. $\hat{TT}(R_f\ 0.28)$ represents the Type I cyclobutane-type thymine dimer. The R_f values are for the solvent used in Table 1.

Figure 2. Separation of Photoproducts on a Column of Sephadex-G10.

A column (0.8 x 35.5 cm) of Sephadex-G10 (40-120μ) was used. The samples were dissolved in and the column was equilibrated with water. 0.5 ml samples were collected every 2 min. T stands for thymine; $\hat{TT}$ for the cyclobutane-type thymine dimer produced when T is U.V. irradiated in frozen solution; and S for the spore photoproduct at R_f 0.35 (see Table 1).


% of Thymine as photoproduct
30
20
10
0
1
2
U.V. dose [ergs/mm² x 10⁻⁵]
Rf 0.35
T̂T [Rf 0.28]

1.


T+T̂T
T̂T
S
T
Cpm
6000
5000
4000
3000
2000
1000
0
S
300
250
200
150
100
50
0
Cpm
10
20
30
40
50
60
Fraction number

2.